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Maize Chlorotic Mottle Machlomovirus Expresses Its Coat Protein from a 1.47-kb Subgenomic RNA and Makes a 0.34-kb Subgenomic RNA

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Analysis of double-stranded RNAs produced in maize plants infected with maize chlorotic mottle machlomovirus (MCMV) and Northern blots of total RNA from infected plants or protoplasts showed two subgenomic RNAs (sgRNAs). Primer extension was used to map these sgRNAs, which are 1.47 and 0.34 kb long. The transcription start sites are nucleotide (nt) 2970 or 2971 for sgRNA1 and nt 4101 for sgRNA2. The 5′ ends of the sgRNAs are similar to one another and to the 5′ end of genomic RNA, and 11 nt sequences immediately upstream of their transcription start sites are similar. The location of the sgRNA1 transcription start site indicates that MCMV expresses a 7-kDa open reading frame (ORF) from nt 2995 to 3202 instead of the predicted 9-kDa ORF from nt 2959 to 3202. In protoplast inoculation experiments, a silent mutation at nt 2965 and a 4-nt change at nt 2959–2962 stopped the synthesis of sgRNA1 and expression of the coat protein ORF, which begins more than 400 nt downstream. Replication of MCMV does not require the expression of any of the ORFs encoded on sgRNA1. SgRNA2 has the potential to encode 2.3-, 2.7-, and 4.6-kDa peptides, but the function, if any, of sgRNA2 is unknown.

INTRODUCTION

Maize chlorotic mottle virus (MCMV) is the only known member of the genus *Machlomovirus*. The host range for MCMV is limited to members of the *Gramineae* with maize (*Zea mays*) as its natural host. Like other members of the family *Tombusviridae*, MCMV has a small icosahedral particle that contains a 4437-nucleotide (nt) plus-sense single-stranded RNA (ssRNA) (Nutter *et al.,* 1989). MCMV is most closely related to panicum mosaic panicovirus (PMV) (Turina *et al.,* 1998) and turnip crinkle carmovirus (TCV) (Nutter *et al.,* 1989). When the sequence of MCMV was initially determined (Nutter *et al.,* 1989), four open reading frames (ORFs) encoding proteins of 32, 50, 9, and 25 kDa were identified, whereas two additional proteins (p111 and p33) were predicted from suppression of the p50 and p9 ORF stop codons, respectively (Fig. 1). As the 5′ proximal ORF, it was suggested that p32, which shows no homology to other proteins in GenBank, was expressed from genomic RNA (gRNA) and that leaky scanning would allow expression of the p50 ORF, which starts 19 nt downstream from the p32 AUG (Nutter *et al.,* 1989). Both p50 and its readthrough protein p111, which contains a GDD box, were predicted to encode the RNA-dependent RNA replicase (RdRp) by comparison with known viral replicase proteins (Nutter *et al.,* 1989). ORFs in the 3′ third of MCMV were predicted to be expressed via subgenomic RNAs (sgRNAs) based on similarity to carmoviruses. The carboxyl end of p9 shows homology to small proteins (7–8 kDa) located in similar positions in carmoviruses (Riviere and Rochon, 1990) and PMV (Turina *et al.,* 1998). An additional small ORF with no start codon encoding an 8-kDa protein, which might be produced as a frameshift fusion near the 3′ end of the p9 ORF, has been predicted (Riviere and Rochon, 1990; Turina *et al.,* 1998). An sgRNA of 1090 nt was identified as the mRNA for the 25-kDa coat protein (CP) (Lommel *et al.,* 1991b). No evidence for a larger sgRNA capable of expressing the p9 ORF and its readthrough product was found when a primer complementary to its first seven codons was used as a probe of Northern blots or for primer extension reactions (Lommel *et al.,* 1991b). Two double-stranded RNAs (dsRNAs) identified as 4.4 and 1.1 kbp and representing genomic RNA (gRNA) and the CP sgRNA were isolated from MCMV-infected plants (Lommel *et al.,* 1991a). It was suggested that p9 might be expressed from a very low abundance sgRNA that did not accumulate to detectable levels in plants.

Members of the family *Tombusviridae* vary in their genome organizations and expression strategies, but viruses from all genera produce sgRNAs. The sgRNAs of many of these viruses have been mapped. PMV produces one sgRNA with a 24-nt leader preceding an 8-kDa ORF, and the second AUG, 397 nt further downstream, begins the CP ORF (Turina *et al.,* 1998). Oat chlorotic stunt avenavirus produces only one sgRNA, which contains the CP ORF and an internal 8-kDa ORF.

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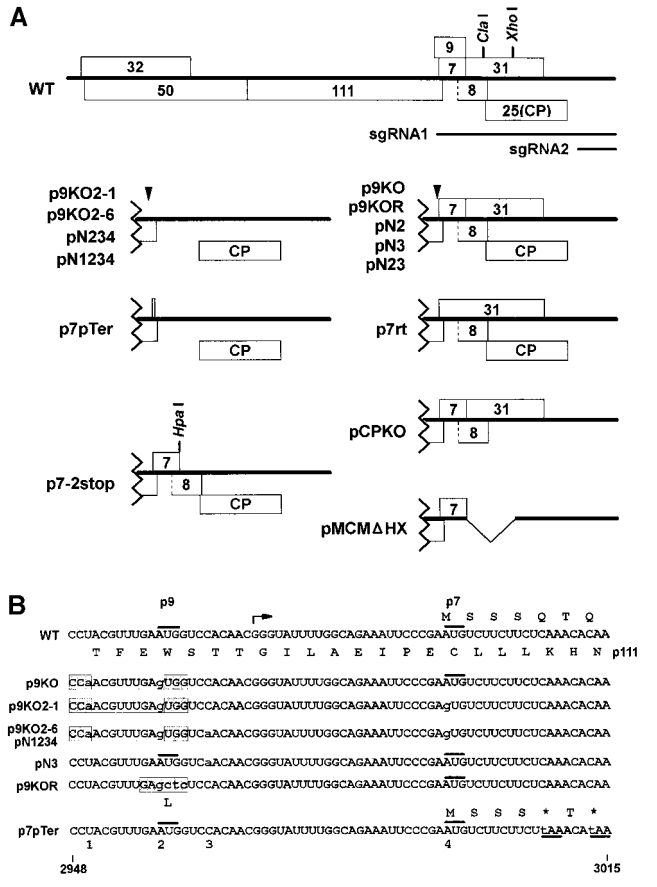

FIG. 1. Genome maps of pMCM41 (WT) and mutant transcripts. (A) The heavy line indicates the genomic RNA, and sgRNAs are marked by thinner lines. The *Cla*I site is 1092 nt from the 3′ end of gRNA. Boxes with numbers indicate the locations of ORFs with the sizes in kDa of their translation products. The p9 ORF, which is in the same reading frame as p7, is placed above p7 for clarity. The 5′ border of the p8 ORF is dashed to indicate it has no start codon. Only the 3′ third of the mutant transcripts is shown. Maps indicate the predicted effects of the mutations on the coding capacity of gRNAs. The region between the *Hpa*I and *Xho*I sites that was deleted in pMCMΔHX is marked by a thin v-line. Arrowheads mark the locations of point mutations shown in detail in part B. (B) Sequences of nt 2948–3015 and the aligned protein sequences for pMCM41 (WT) and some mutant transcripts. Mutated bases are in lower case, AUGs are overlined, and stop codons are underlined. The 5′ ends of the p9 and p7 ORFs are labeled. The protein sequence of p111 was unchanged for all mutations except p9KOR, which shows only the changed residue. The *Xcm*I sites and *Sac*I site that were introduced into the p9KO series and p9KOR, respectively, are marked by gray boxes. The locations of the four bases mutated individually or in combinations (pN1234 series) are marked below the alignment so the sequences of pN2, pN23, and pN234 can be visualized. The bent arrow indicates the location and direction of sgRNA1 synthesis.

The 5′ end of this sgRNA has been mapped to 141 nt upstream of the CP AUG, and three very small ORFs are located upstream of the CP ORF (Boonham *et al.,* 1998). Carmoviruses, necroviruses, tombusviruses, and aureusviruses produce one sgRNA with the CP gene as the most 5′ ORF and an additional sgRNA for expressing other ORFs either upstream or downstream of the CP ORF. Mapping of sgRNAs from carnation mottle carmovirus (Carrington and Morris, 1986), TCV (Carrington *et al.,* 1987; Wang and Simon, 1997), and saguaro cactus carmovirus (Weng and Xiong, 1997) showed the larger sgRNAs have untranslated leaders of 19–26 nt, whereas the smaller CP sgRNAs have untranslated leaders of 137 nt. No sgRNAs were detected with cardamine chlorotic fleck carmovirus (Skotnicki *et al.,* 1993), but two sgRNAs were found for galinsoga mosaic carmovirus (Morris and Carrington, 1988), melon necrotic spot carmovirus (Riviere and Rochon, 1990), and cowpea mottle carmovirus (Kim and Bozarth, 1992). The untranslated leaders of tobacco necrosis necrovirus-A (TNV-A) (Meulewaeter *et al.,* 1992), tobacco necrosis necrovirus-D (TNV-D) (Offei and Coutts, 1996), and olive latent necrovirus (Grieco *et al.,* 1996) are 36–48 nt long on the large sgRNAs and 90–152 nt long on the smaller CP sgRNAs. Both sgRNAs of tomato bushy stunt tombusvirus (TBSV) (Hillman *et al.,* 1989), cymbidium ringspot tombusvirus (CyRSV) (Grieco *et al.,* 1989), cucumber necrosis tombusvirus (CNV) (Rochon, *et al.,* 1991), and artichoke mottle crinkle tombusvirus (Tavazza *et al.,* 1994) have been mapped and have short untranslated leader regions 13–32 nt long. Although two sgRNAs were detected in pothos latent aureusvirus (Sabanadzovic *et al.,* 1995) and cucumber leaf spot aureusvirus (Miller *et al.,* 1997), only the transcription start site of the large sgRNA from cucumber leaf spot aureusvirus has been mapped to 30 nt upstream of the CP AUG. Dianthoviruses, the only genus with bipartite genomes, produce one sgRNA to express CP that is encoded on RNA1. The transcription start site of the sgRNA of red clover necrotic mosaic dianthovirus has been mapped to 62 nt upstream of the CP ORF (Zavriev *et al.,* 1996).

I have been using mutants of infectious transcripts of MCMV to investigate the functions of the proteins encoded in MCMV. Some of the mutants have characteristics inconsistent with the previous predictions of the expression strategy of MCMV. This has lead to a reanalysis of the sgRNAs produced by MCMV using dsRNA analysis, Northern blot hybridization, primer extension, and 5′ rapid amplification of cDNA ends (RACE). These experiments and analyses of additional mutant transcripts in maize protoplast replication assays suggest a different sgRNA profile for MCMV that clarifies the expression mechanisms for the ORFs in the 3′ third of the genome. These results help elucidate the relationship of MCMV to the other members of the family *Tombusviridae.*

RESULTS

Point mutations more than 400 nt upstream of the CP ORF affect its expression

To help determine gene functions, mutations were introduced into pMCM41, an infectious cDNA clone of

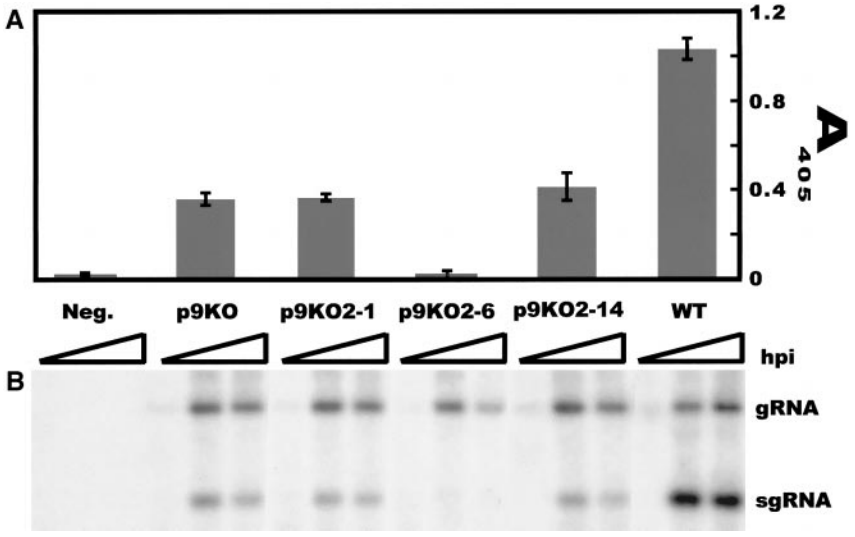

FIG. 2. CP and viral RNA accumulation in protoplasts inoculated with WT transcripts or transcripts with mutations between nt 2950 and 2995 of MCMV. BMS protoplasts (1.4×10^6) were inoculated with 10 μg of transcript RNA or tRNA (negative control). Samples were collected at 0, 24, and 48 h p.i. for CP and RNA analysis. Sample names for panels A and B are placed between the panels. (A) CP accumulation at 48 h p.i. assayed by PAS-ELISA. Gray bars represent the average absorbance at 405 nm with error bars marking the range. (B) Northern blot of viral RNAs. Total RNA from equivalent volumes of samples was separated by denaturing agarose gel electrophoresis and analyzed by Northern blot hybridization with a probe complementary to the 3′ 159 nt of MCMV. The location of gRNA and the large sgRNA are indicated on the right.

MCMV (Scheets *et al.,* 1993), using *in vitro* mutagenesis (Kunkel *et al.,* 1987). Many mutations introduced a restriction site to simplify screening. Two to four clones with the correct restriction sites were then sequenced near the mutation site and inoculated to Black Mexican Sweet (BMS) maize protoplasts to determine their ability to produce viral RNAs and CP. Clones with the same introduced mutations and the same phenotype in protoplasts were considered identical with no other mutations elsewhere in the MCMV genome. Several mutations were introduced into the 3′ third of the genome. An *Xcm*I restriction site was added by mutating nt 2950 (T to A) and nt 2959 (A to G), which changed the start codon of the predicted p9 ORF from ATG to GTG (nt 2959–2961) in p9KO (Fig. 1B). Mutagenesis of p9KO at nt 2995 changed an additional in-frame ATG to GTG, removing the only other start codon preceding the CP ORF (nt 3384–3386) to produce p9KO2 (Fig. 1). All three base changes produce silent mutations in the overlapping RdRp ORF (Fig. 1B). When tested in BMS protoplast assays, transcripts from p9KO consistently produced lower accumulations of CP, and lower concentrations of the large sgRNA were detected on Northern blots at all time points compared with wild-type (WT) infections (Fig. 2 and data not shown). Three of four independent clones of p9KO2 (p9KO2-1, p9KO2-14, and p9KO2-17) contained the expected base changes and replicated in protoplasts similar to p9KO (Fig. 2; p9KO2-17 not shown). A fourth clone, p9KO2-6, contained the expected three base changes and an additional C-to-A mutation at nt 2965, which lies between the two mutated ATG codons. Transcripts from p9KO2-6 replicated in protoplasts but did not produce the large sgRNA or CP (Fig. 2). The C-to-A mutation at nt 2965 is a silent mutation in the RdRp ORF and would produce an His-to-Asn change at residue 3 of p9 (Fig. 1B). Because p9KO and all the p9KO2 cDNAs have only silent mutations in the RdRp ORF, and these mutations are more than 400 nt upstream of the CP ORF, it seemed likely that the lack of sgRNA and CP synthesis in p9KO2-6 and the decrease in sgRNA and CP synthesis in p9KO, p9KO2–1, p9KO2-14, and p9KO2-17 were due to changes in a *cis*-acting RNA sequence such as an sgRNA promoter. The location of the mutations that affected sgRNA synthesis lead to the hypothesis that MCMV expresses a p7 ORF not a p9 ORF, and the upstream in-frame AUG was part of the sgRNA promoter. Experiments were planned to test this hypothesis.

Determining sgRNAs of MCMV by dsRNA and Northern blot hybridization

The ability of an MCMV infection to produce an sgRNA large enough to express the ORFs upstream of the CP ORF was reexamined. DsRNA preparations from maize plants infected with MCMV consistently produced three bands that migrated with estimated sizes of 4.4, 1.5, and 0.35 kbp compared with DNA standards (Fig. 3A). When total RNA from noninfected or MCMV-infected plants was separated on a denaturing gel and compared with transcript RNA and virion RNA (vRNA), hybridization with a 159-nt 3′ terminal probe produced three bands in RNA from MCMV-infected plants that migrated at 4.4, 1.49, and 0.33 kb compared with ssRNA standards (Fig. 3B). Faint bands corresponding to 1.75-, 1.49-, and 0.33-kb RNA

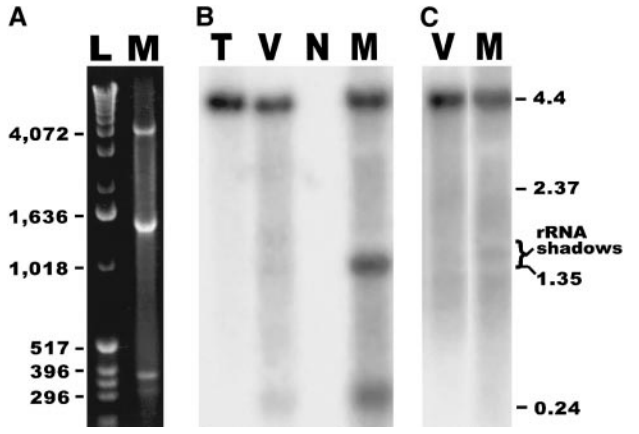

FIG. 3. Genomic and subgenomic RNAs from MCMV-infected maize. Total RNA and dsRNA were isolated from MCMV-inoculated or noninoculated maize plants. (A) Photograph of ethidium bromide-stained nondenaturing agarose gel containing dsRNAs isolated from MCMV-infected maize plants (M) and dsDNA ladder (L). The sizes (bp) of some of the DNA markers are indicated on the left. (B) Phosphor image from a Northern blot hybridized with a probe complementary to the 3′ 159 nt of MCMV. Transcript RNA from pMCM41 (T), MCMV virion RNA (V), and total RNA isolated from maize plants that were mock-inoculated (N) or inoculated with MCMV (M) was separated by denaturing agarose gel electrophoresis before transferring the RNA to a membrane for hybridization. (C) Phosphor image from a duplicate Northern blot hybridized with a probe complementary to the 5′ 1.1 kb of MCMV. Lanes are labeled as in part B. The location and sizes (in kb) of a ssRNA size ladder are indicated on the right, and the bracket marks the location of negative shadows caused by rRNA.

were found in vRNA (Fig. 3B). The 1.75-kb band, which was not consistently detected in different vRNA preparations (data not shown), may denote a gRNA degradation band [see Fig. 6B, WT at 51 h postinoculation (p.i.)]. Hybridization of a duplicate blot with a 1.1-kb 5′ terminal probe showed only a gRNA band, indicating that the 0.33-kb RNA band was not due to degradation (Fig. 3C). Bands in the total RNA lane (Fig. 3B) and in total RNA from four additional infected plants were quantified by phosphor imaging. The 1.49-kb RNA, sgRNA1, was present at 53–158% of gRNA concentrations. This was similar to the 45–133% range determined for the 0.33-kb RNA (sgRNA2), but there was no correlation between the concentrations of the two sgRNAs within a plant (data not shown).

Mapping the sgRNAs of MCMV

The 5′ end of sgRNA1 was determined using primer extension from an oligonucleotide (p7sg RO, Table 1) complementary to nt 3028–3045, a region 33 nt downstream from the second AUG in the p9 ORF. SgRNA2 was mapped using a primer (3′sg RO, Table 1) complementary to nt 4138–4155, which was expected to hybridize about 50 nt downstream from its 5′ end. The primer originally used to map the CP sgRNA (p25 RO, Table 1) complementary to nt 3384–3403, was used in an attempt to confirm the location of this sgRNA. Each primer extension reaction was performed two or three times on

TABLE 1

Summary of Oligonucleotides Used in the Study

Application/ Construct	Oligonucleotide name[a]	Position in MCMV	Sequence[b] (5′ to 3′)	Enzyme	Polarity[c]
Primer run-off	p7sg RO	3028–3045	TTGTCTGCGGGCTACGTC		−
	p25 RO	3384–3403	GACCGGCTACTTGCCGCCAT		−
	3′sg RO	4138–4155	GGACTCTGCCAGAAGGAC		−
5′ RACE	OSU4	4424–4437	gatcaagcttGGGCCGGAAGAGAG		−
	GSP1	4388–4408	CCCGTTCTACTCCGTTGAGTT		−
Site-directed mutagenesis					
p9KO	p9KO	2943–2964	GGACCCCaACGTTTGAgTGGTC	*Xcm*I	+
p9KOR	p9KOR	2946–2967	CCCC(a/T)ACGTTTGAgctcTCCAC	*Sac*I	+
pN1234	p9KO:C → A	2950–2972	(a/T)ACGTTTGAgTGGTCaACAACGG		+
pN234					
pN23					
pN3	2965:C → A	2952–2972	CGTTTGAATGGTCaACAACGG		+
p9KO2	p7KO	2982–2999	CAGAAATTCCCGAgTGTC	*Ava*I	+
p7pTer	p7pTer	2997–3019	GTCTTCTTCTtAAACAtAATCCC		+
p7rt	p7rt	3186–3206	CTTCAATTTCAAtTGgGCTGG	*Mun*I	+
p7-2stop	p7-2stop	3189–3212	CAATTTCAACTGAGgTtaAcTGTG	*Hpa*I	+
pCPKO	CPKO	3370–3390	GATCGACAcACACAgTGGCGG	*Dra*III	+

[a] All oligonucleotides were made by the OSU Recombinant DNA/Protein Resource Facility except for OSU4 (OU Health Sciences Resource Center), p9KO and CPKO (Oligos Etc., Inc.), and p25 RO (gift from S. A. Lommel, North Carolina State University).

[b] Bases in lowercase are non-MCMV sequence, and underlined bases show the recognition sites for the restriction enzymes listed in the next column. (a/T) represents a mixed base with mutant or WT bases in the same position.

[c] Polarity relative to gRNA.

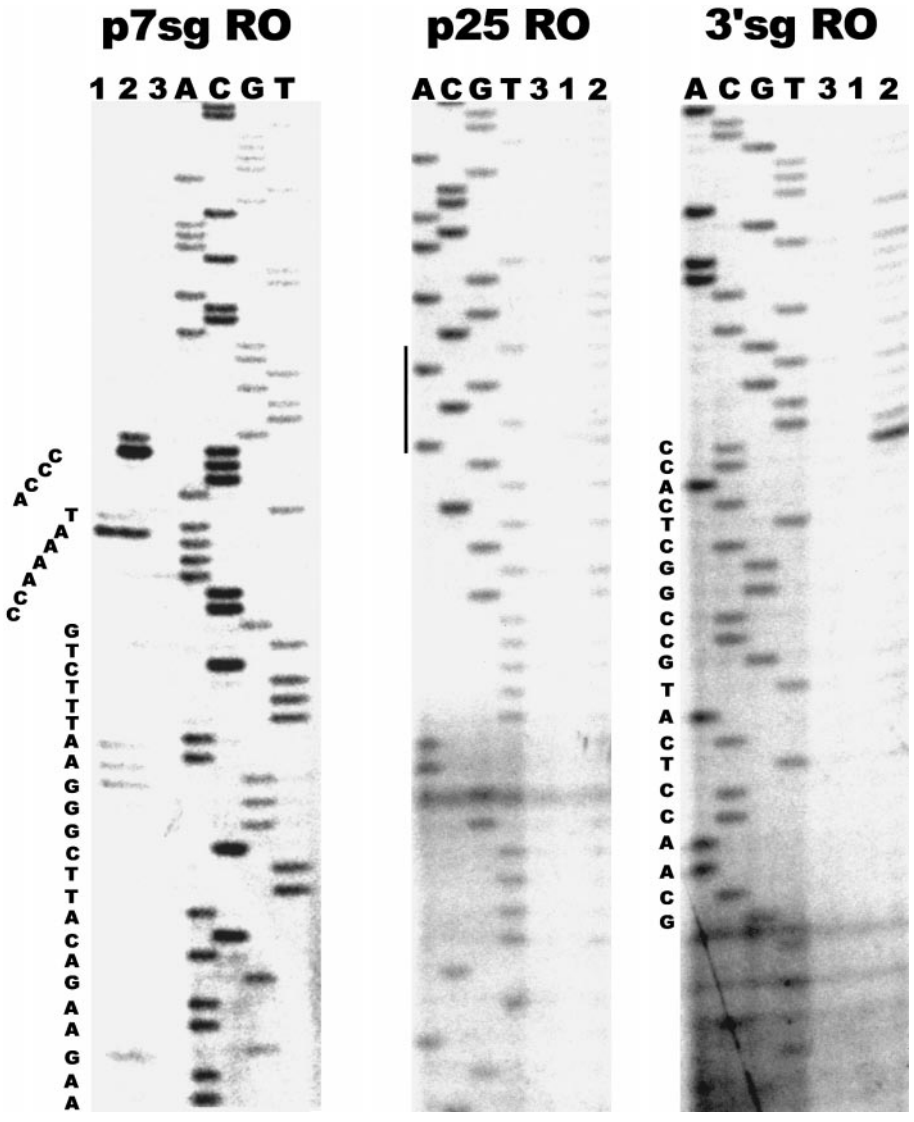

FIG. 4. Primer extension mapping of the 5′ ends of MCMV sgRNAs. Primer extension reactions were performed using the three indicated ^{33}P-labeled primers (Table 1) on total RNA from mock-inoculated maize plants (lanes 1), total RNA from MCMV-inoculated maize plants (lanes 2), or pMCM41 transcript RNA (lanes 3). Dideoxy-termination sequencing ladders of pMCM41 DNA using the same primers were run next to each set of reactions. The sequences complementary to the 5′ ends of sgRNA1 (p7sg RO) and sgRNA2 (3′sg RO) are shown next to their sequencing ladders, and the vertical line marks the *Cla*I site marked on the genome map in Fig. 1.

multiple total RNA samples from noninfected and infected plants or protoplasts. Discrete, MCMV-specific primer extension products were detected in total RNA from MCMV-infected plants when p7sg RO and 3′sg RO primers were used (Fig. 4, lanes 2). Transcript RNA did not produce a strong stop band with any of the three primers, indicating that the p7sg RO and 3′sg RO bands were not due to termination from strong secondary structure in the RNA. Primer extension from p7sg RO produced medium and heavy bands corresponding to initiation at nt 2970 and 2971 of MCMV. A doublet of these bands was also observed when primer extension was performed on RNA from infected protoplasts (data not shown). This suggested either that sgRNA synthesis can initiate at either nt or that a 1467-nt-long sgRNA initiating at nt 2971 might be capped with m^{7}G since reverse transcription of capped viral RNAs can produce a 1-nt extension past the 5′ end (Ahlquist and Janda, 1984; Gupta and Kingsbury, 1984). The major sgRNA1 has a 24-nt untranslated region preceding the p7 AUG, and the second AUG, 353 nt further downstream, begins the CP ORF. A maize-specific band 5 nt shorter than the major MCMV-specific band was consistently detected with p7sg RO primer. The 3′sg RO primer extension product corresponded to an RNA initiating at nt 4101. Although there was a lighter band 1 nt larger than the major extension product from this primer, there were bands of approximately equal intensity at several additional positions above and below the major extension product. Primer extension using RNA from infected protoplasts

showed a stronger contrast between the intensities corresponding to nt 4101 and 4100 (data not shown), suggesting that sgRNA2 is 337 nt long and uncapped. There are three nonoverlapping ORFs on sgRNA2 capable of coding for peptides of 2.7, 2.3, and 4.6 kDa, none of which show homology to other proteins in GenBank. The start codons of the three ORFs are 12, 107, and 180 nt downstream from the 5′ end of sgRNA2. No MCMV-specific strong extension product could be detected using p25 RO in this experiment (Fig. 4) or in a separate primer extension experiment (data not shown). Primer extension mapping showed that the size of sgRNA1, the sgRNA responsible for CP synthesis, is 1467 or 1468 nt long and that sgRNA2 is 337 nt long.

5′ RACE of sgRNA2

Several members of the family *Tombusviridae* produce small defective interfering RNAs of a size similar to sgRNA2. To confirm the start site of sgRNA2, 5′ RACE was performed on total RNA from MCMV-infected plants or dsRNA isolated from a nondenaturing agarose gel. Both sources of RNA produced a PCR band of the expected size, ~350 bp (data not shown). The PCR products were ligated into a vector, and individual clones were selected for restriction fragment sizing and sequencing. Seven cDNAs had inserts of 350 bp, whereas six cDNAs had slightly smaller inserts. Three 350-bp cDNAs and two smaller cDNAs were sequenced, and all were colinear with the 3′ end of MCMV (data not shown). A 1-nt change was found in two cDNAs compared with the reported sequence of MCMV at nt 4202 (A instead of G) and nt 4208 (C instead of T). The C-to-T mutation removes the start codon at the beginning of the 2.3-kDa ORF, which would produce a slightly smaller ORF initiating 6 nt downstream. The 5′ ends of the 350-bp cDNAs started at nt 4101, confirming that the small RNA is an sgRNA (data not shown). The 5′ ends of the shorter cDNAs started 29 or 64 nt downstream of the sgRNA2 start site (data not shown). The shorter cDNAs either represent premature termination of reverse transcription during RT-PCR or represent full-length RT-PCR products synthesized from degraded RNA. The large number of light bands from primer extension with 3′sg RO (Fig. 4) suggests that there was degraded RNA of this size range present in the RNA preparations.

Identifying parts of an sgRNA promoter near nt 2959

Because the initiation site of sgRNA2 is located between the two AUGs in the p9 ORF, additional mutants were used to test whether the region between nt 2959 and nt 2995 contained an sgRNA promoter and whether the upstream proteins encoded on sgRNA1 were required for expression of CP. In p9KOR, the start codon of p9 was changed from ATGG to GCTC (nt 2959–2962), introducing a *Sac*I site that also changed the coding in the RdRp from Trp to Leu (Fig. 1B). The C-to-A mutation (nt 2965) detected in p9KO2-6 was introduced into p9KO2-1 to reproduce the four base changes (pN1234) (Fig. 1B). The same point mutation was introduced into pMCM41 alone (pN3) or in combination with two of the point mutations in p9KO2–1 (pN23 and pN234). An additional mutant pN2 (ATG to GTG at nt 2959–2961) was obtained from this mutagenesis reaction.

Stop codons were added or removed downstream of the p9 and p7 start codons to test whether expression of proteins from this region was required for CP expression (Figs. 1A and 1B). By changing nt 3007 and 3013, two premature termination codons (TAA) replaced the fifth and seventh codons (CAA) of the p7 ORF in p7pTer, leaving the encoded RdRp unchanged. The insertion of an *Hpa*I site (GTTAAC replacing CTGGAG at nt 3203–3208) just downstream of the p7 stop codon (nt 3199–3201) added a second in-frame stop codon in p7-2stop, which should not allow the production of any readthrough protein. In p7rt, the p7 opal codon was changed to a Trp codon (CTGA to TTGG at nt 3198–3202) when an *Mun*I site was introduced, producing a 31-kDa ORF. To stop the expression of CP without making mutations near the sgRNA1 transcription start site, two additional mutants were constructed (Fig. 1A). In pCPKO, changing two bases introduced a *Dra*III site, removing the CP start codon (TACACAATG to CACACAGTG at nt 3378–3386), and pMCMΔHX is missing nt 3206–3579, which removes the region surrounding the beginning of the CP ORF.

BMS protoplasts were inoculated with WT and mutant transcripts and tested for production of CP and viral RNAs. All the mutant transcripts were capable of replicating in protoplasts. No CP could be detected (Figs. 5A and 6A), and sgRNA1 was not produced (Figs. 5B and 6B) in infections of any transcripts containing the C-to-A mutation at nt 2965 (pN3, pN23, pN234, pN1234). This indicated that the C-to-A mutation was sufficient to reproduce the characteristics of p9KO2-6. The same phenotype was seen for p9KOR, indicating that the amino acid change in the RdRp of p9KOR did not greatly affect its function and that nt 2959–2962 are part of the promoter (Fig. 5). Both sgRNA1 and CP were produced by pN2 at levels less than WT (Fig. 6) but higher than p9KO2-1 (Figs. 2 and 5), suggesting that both nt 2950 and nt 2959 are less critical positions in the promoter for sgRNA1. As expected, pMCMΔHX did not produce CP (Fig. 5A), but gRNA and sgRNA1 slightly smaller than WT were detected on the Northern blot (Fig. 5B). As well as readily detectable sgRNA1, a very small but reproducible CP signal could be detected with pCPKO (Fig. 5A), which was more evident in experiments lasting 72 h p.i. (data not shown). This could be caused by very low initiation from the mutated (GUG) start codon or from initiation at the next AUG, which is also in the CP ORF. Infec-

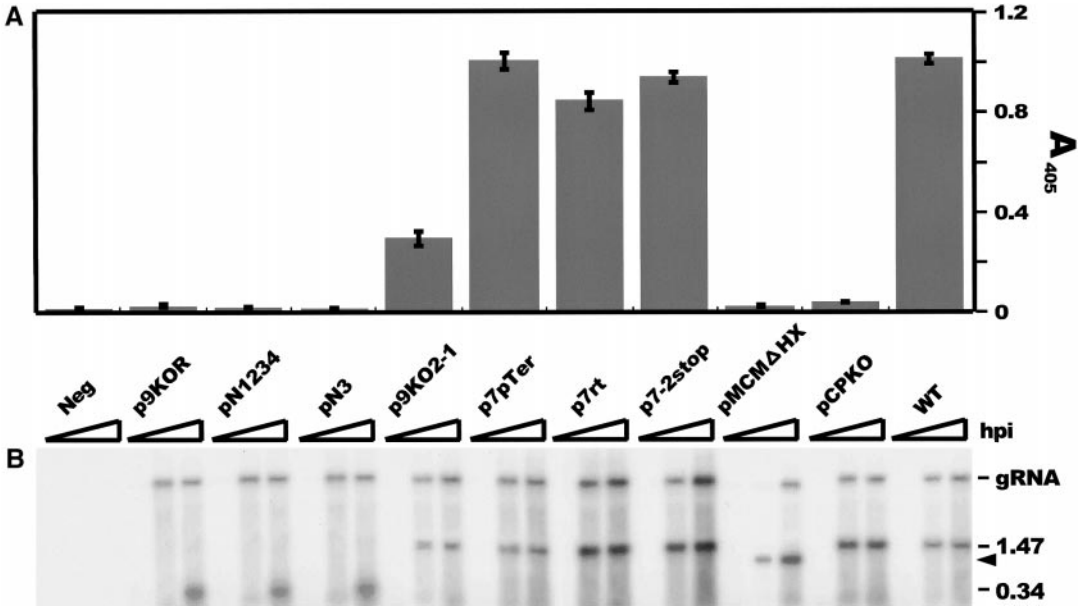

FIG. 5. CP and viral RNA accumulation in protoplasts inoculated with WT and mutant transcripts of MCMV. BMS protoplasts (1.25×10^6) were inoculated with 15 μg of transcript RNA or tRNA and were assayed as in Fig. 2. (A) CP accumulation at 48 h p.i. (B) Northern blot of viral RNAs. The location of gRNA and the two sgRNAs are indicated on the right. The arrowhead marks the migration size of sgRNA1 from the deletion clone pMCMΔHX.

tions from mutants not producing CP generally accumulated levels of gRNA lower than WT or other mutants producing CP (Figs. 5B and 6B). There was some variability in the amount of full-length transcript taken up by the protoplasts as determined by quantifying the 0-h lanes with the PhosphorImager. The levels of gRNA at 48 h for Fig. 5 or 28 h for Fig. 6 did not correlate with the levels of input RNA but instead correlated with the presence of CP (data not shown). The lower levels of gRNA from mutants not expressing CP were probably caused by increased degradation of the unencapsidated RNA. This was especially noticeable in Fig. 6B, where the 51-h lanes were heavily degraded compared with 28-h lanes.

The addition or removal of in-frame stop codons in the p7 ORF or its readthrough region (p7pTer, p7-2stop, p7rt) did not stop the production of CP or sgRNA1 (Fig. 5), indicating that expression of proteins from this region

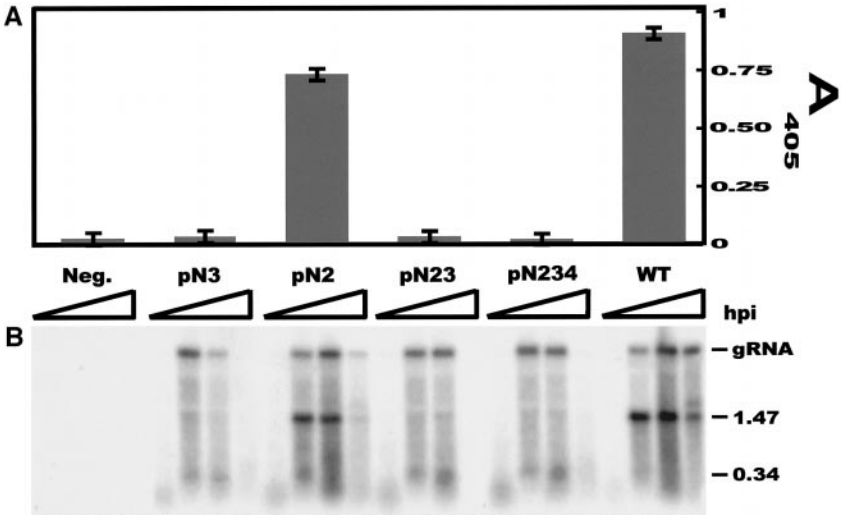

FIG. 6. CP and viral RNA accumulation in protoplasts inoculated with WT transcripts or transcripts with mutations between nt 2959 and 2995 of MCMV. BMS protoplasts (1.4×10^6) were inoculated with 15 μg of transcript RNA or tRNA and were assayed as in Fig. 2 except that sampling times were 0, 17.5, 28, and 51 h p.i. (A) CP accumulation at 51 h p.i. (B) Northern blot of viral RNAs. The location of gRNA and the two sgRNAs are indicated on the right. Note the rRNA shadows (see Fig. 3) in pN3, pN23, and pN234 that cause faint artefactual bands slightly larger than sgRNA1.

was not required for expression of CP. These experiments showed that several mutations from nt 2950 to 2965 affect the promoter of sgRNA1, and there was a direct correlation between the levels of CP and sgRNA1.

DISCUSSION

MCMV does not require any of the proteins encoded in the 3′ third of its genome for replication in BMS protoplasts. This is not surprising because the ORFs upstream and downstream of the suppressible stop codon encoding the RdRp are the only viral ORFs found necessary for replication in related viruses such as TCV (Hacker *et al.,* 1992), CyRSV (Dalmay *et al.,* 1993), TBSV (Scholthof *et al.,* 1995), TNV-D^{H} (Molnár *et al.,* 1997), or artichoke mottle crinkle virus (Molinari *et al.,* 1998). All of the ORFs downstream of the RdRp ORFs of CNV (Johnston and Rochon, 1995) and pothos latent aureusvirus (Rubino and Russo, 1997) are also dispensable for replication. MCMV does express p7 and its p31 readthrough protein because replication competent transcripts with mutations in these ORFs do not produce an infection in plants (manuscript in preparation).

MCMV was found to produce two sgRNAs of 1.47 and 0.34 kb, distinct from the size previously reported for a single sgRNA (Lommel *et al.,* 1991a, 1991b). This difference does not reflect a difference in isolates of MCMV. The MCMV-Kansas isolate used for genome sequencing (Nutter *et al.,* 1989), characterization of MCMV (Lommel *et al.,* 1991a), determination of a single sgRNA (Lommel *et al.,*1991b), construction of pMCM41 (Scheets *et al.,*1993), and in this report originated from the same source. In this report, the sizes of the dsRNA bands detected on nondenaturing gels and the ssRNA bands detected on Northern blots closely correspond to the sizes determined from primer extension experiments for sgRNA1 and sgRNA2. Because no RNA bands corresponding to a size of 1.1 kb were detected and no MCMV-specific primer extension product was made from the p25 RO primer, it was concluded that an sgRNA of 1090 nt is not synthesized. In all protoplast inoculation experiments, whether using virions, vRNA, WT transcripts, or mutant transcripts with intact CP ORFs, CP expression correlated with the synthesis of sgRNA1 (Figs. 2, 5, and 6, Scheets *et al.,* 1993; and data not shown). CP expression did not require the expression of p7 or its readthrough product, since the addition or removal of stop codons within p7 or its readthrough ORF did not stop or decrease the expression of CP. Although it is not currently known whether the p8 ORF of MCMV is expressed, the mutation in p7pTer would not allow the production of any p7 + p8 frameshift product, so the expression of this putative ORF was not necessary for CP synthesis either. If MCMV expresses the p9 ORF, it would require internal ribosome entry on gRNA (see Maia *et al.,* 1996, for a review of viral gene expression) or a larger sgRNA for which there is no evidence (Lommel *et al.,* 1991b).

In earlier work, primer hybridization and primer extension were used to detect MCMV sgRNAs (Lommel *et al.,* 1991b). In primer hybridization to Northern blots, the p25 RO primer would be capable of detecting sgRNA1 because the complete 20-mer would be able to hybridize to the RNA. These workers were unable to detect sgRNA1 with an upstream probe using hybridization conditions designed for a 20-mer because their probe, which is complementary to the first seven codons of the p9 ORF (nt 2959–2078), could hybridize to only the first 8 nt of sgRNA1. Primer extension from the same oligonucleotide would be unable to detect sgRNA1 either.

The 5′ ends of the sgRNAs of MCMV are similar to the 5′ end of gRNA with 10 of 15 nt identical when 2-nt gaps are included in the alignments for the sgRNAs (Fig. 7A). Similarities between the 5′ ends of gRNAs and their sgRNAs have also been noted for many other plant viruses, such as brome mosaic bromovirus (Marsh and Hall, 1987), alfalfa mosaic almovirus (van der Kuyl *et al.,* 1991), cucumber mosaic cucumovirus (Boccard and Baulcombe,1993), barley yellow dwarf luteovirus-PAV (Kelly *et al.,* 1994), southern bean mosaic sobemovirus (Hacker and Sivakumaran, 1997), many of the poleroviruses (for a review, see Miller *et al.,* 1995), and many members of the family *Tombusviridae* mentioned in the introduction.

In the 21 nt regions immediately upstream of the sgRNA transcription start sites in MCMV, 8 of 11 bases are identical (Fig. 7A). Alignment places a gap between the upstream consensus sequence and the 5′ end of sgRNA2. Only one extension product is found, indicating only one initiation site for sgRNA2. In contrast, the alignment places the C at nt 2970, which corresponds to initiation at the weaker primer runoff signal for p7sg RO (Fig. 4), as an extra base between the upstream consensus sequence and the 5′ end of the stronger signal for sgRNA1. This suggests that the two primer extension products detected for sgRNA1 are more likely produced from sgRNA initiation occurring at two sites rather than by capping of an sgRNA1 that only initiates at nt 2971. Insertions or deletions in the putative spacer region of the brome mosaic bromovirus sgRNA promoter can alter the choice of initiation site by its RdRp *in vitro* (Stawicki and Kao, 1999). All except one of the mutated bases that reduced or eliminated sgRNA1 and CP synthesis changed bases in the 11-nt region of similarity, showing that it is a critical part of the sgRNA1 promoter. Mutants pN2, p9KO, and p9KO2-1 have a mutation at a conserved A in this region, and p9KO and p9KO2-1 have a second mutation at a conserved U 9 nt further upstream that reduced the levels of sgRNA1 and CP synthesis. Complete loss of sgRNA1 synthesis occurred with p9KOR where three of the four mutated bases are conserved. The single C-to-A change in pN3, the mutation located

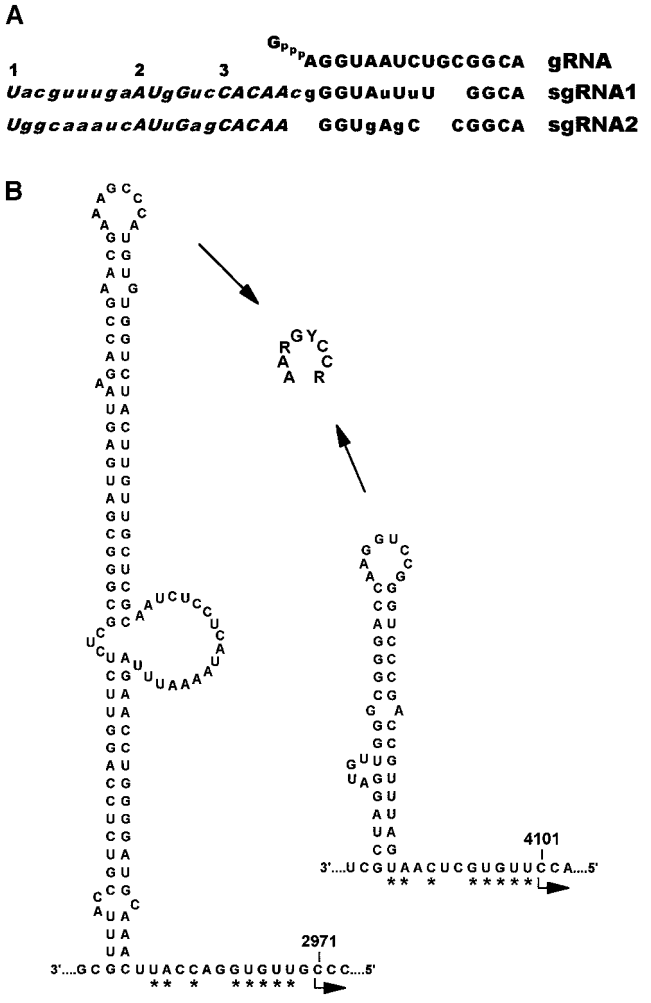

FIG. 7. Sequences surrounding the sgRNA start sites and the 5′ end of MCMV. (A) Alignment of gRNA sequences showing similarities between the 5′ ends of gRNA and sgRNAs. Identical bases are in uppercase, and 2-nt gaps have been added for maximum alignment. Sequences upstream of the sgRNA transcription start sites are italicized, and the location of mutated bases in pN1234 are marked. (B) Computer-predicted RNA secondary structures for minus-RNA near the sgRNA transcription start sites. Asterisks mark the 8 conserved nt near the transcription start sites, which are marked by bent arrows. The numbers refer to positions in gRNA. Large arrows point to the consensus sequence of the loops.

closest to the sgRNA1 transcription start site, also completely eliminated sgRNA1 and CP synthesis.

RNA secondary structure was predicted for the minus-strand of the regions surrounding the sgRNA transcription start sites. Folding of the RNA corresponding to the region upstream of the sgRNA1 transcription start site produced a long stem-loop structure 14 nt from the transcription start site (Fig. 7B). An analogous region upstream of the sgRNA2 transcription start site formed a shorter stem-loop structure (Fig. 7B). Both stems start at or near the end of the 11-nt conserved regions. Although no significant sequence similarity was found in the stems, the 8-nt loops at the top of the stems have a consensus sequence of 5′-RCCYGRAA-3′. These stem-loop structures may be part of the sgRNA promoters. The promoter regions for TCV sgRNAs have been mapped to 90 nt upstream and 6 or 4-nt downstream of the transcription start sites of the 1.7- and 1.45-kb sgRNAs, respectively (Wang and Simon, 1997). There is 40% sequence similarity between the promoters, and minus-strand promoter regions fold into similar large stem-loop structures. Recently, the promoter for the 1.45-kb sgRNA has been more precisely mapped to the bottom of the stem corresponding to a 21-nt stem-loop region and a 9-nt single-stranded region adjacent to the transcription start site (Wang *et al.,* 1999). The promoter for sgRNA2 of CNV has been mapped to approximately 20 nt upstream and 6 nt downstream of the initiation site (Johnston and Rochon, 1995). For TBSV, sgRNA2 requires sequences immediately upstream of the initiation site and a *cis*-acting sequence ~1000 nt upstream (Zhang *et al.,* 1999), whereas expression of red clover necrotic mosaic dianthovirus sgRNA requires sequences immediately upstream of the initiation site and a *trans*-acting sequence from RNA2 (Sit *et al.,* 1998).

MCMV produces only one large sgRNA capable of expressing the 7- to 31-kDa proteins encoded in the 3′ third of its genome. Initiation of translation from the first AUG of sgRNA1 would produce p7, and suppression of the opal stop codon in this frame would allow production of the 31-kDa readthrough protein. A (+1) frameshift near the end of the p7 ORF would produce a 15-kDa fusion protein (p7 + p8). CP may be expressed by leaky scanning past the p7 AUG, or it might be expressed by internal ribosome entry. PMV also encodes a large number of ORFs on its single sgRNA (Turina *et al.,* 1998). For both MCMV and PMV, the CP AUG is the second AUG on the sgRNA. This similarity is not surprising since MCMV and PMV are more closely related to each other than to any other viruses (Turina *et al.,* 1998). Recently, four viruses that infect geranium have been proposed as members of a new genus provisionally designated *Pelarspovirus* within the family *Tombusviridae* (R. Jordan, personal communication). These viruses have genome organizations similar to carmoviruses except that the second small ORF has no start codon, suggesting that it might be expressed as a (−1) frameshift fusion with the first ORF. These viruses make only one sgRNA, which is large enough to encode the two small ORFs and the downstream CP ORF (R. Jordan, personal communication). Thus, there are now two general categories for CP expression within the family *Tombusviridae*: viruses that express their CP from its own sgRNA, and viruses that express their CP as a downstream ORF of a larger sgRNA.

Using a 159-nt probe corresponding to the 3′ end of the genome, it was found that sgRNA2 is present at fairly high concentrations in infected plants. In protoplast inoculations with transcripts containing mutations in the

sgRNA1 promoter, higher-than-normal concentrations of sgRNA2 accumulated (Figs. 5B and 6B). For TCV, mutations that inactivated the 1.45-kb sgRNA promoter caused an increased production of the 1.7-kb sgRNA, which is normally produced in lower abundance than the 1.45-kb sgRNA (Hacker *et al.,* 1992; Wang and Simon, 1997).

SgRNA2 has the potential to encode three peptides from three nonoverlapping ORFs, but it is not known whether any of these ORFs are translated. All sequenced tombusviruses have a small ORF at their 3′ ends capable of encoding conserved peptides 32–69 amino acids long, designated pX (Boyko and Karasev, 1992). Although a sequence that might be part of an sgRNA promoter is found upstream of the pX ORFs (Boyko and Karasev, 1992), only CNV produces a viral RNA of the appropriate size (~350 nt for CNV) in infected plants and protoplasts (Johnston and Rochon, 1995). Mutational analyses of CyRSV (Dalmay, *et al.,* 1993) and TBSV (Scholthof and Jackson, 1997) suggested that expression of their pX ORF is not necessary for replication but that this region contains *cis*-acting RNA sequences that affect viral RNA replication and, for TBSV, affects viral accumulation in a host-specific manner (Scholthof and Jackson, 1997). Barley yellow dwarf luteovirus-PAV makes a 329-nt sgRNA3 with no coding capacity (Kelly *et al.,* 1994; Koev *et al.,* 1999). This sgRNA was produced in large amounts in infected tissue and was detected at much lower levels in virions, suggesting that it is poorly encapsidated. It was speculated that sgRNA3 might have a regulatory function (Kelly *et al.,* 1994). Further work will be needed to determine the function of sgRNA2 and to determine the translation strategies for sgRNA1 in MCMV infections.

MATERIALS AND METHODS

Construction of mutant cDNAs

In vitro mutagenesis was performed on pMCM41 (Scheets *et al.,* 1993), a cDNA clone of the infectious transcript of MCMV-Kansas, using the method of Kunkel *et al.* (1987) and oligonucleotides listed in Table 1. Additional mutations were introduced into p9KO and p9KO2-1 (Fig. 1B) using the same method. The cDNA pMCMΔHX was produced by digesting p7-2stop (Fig. 1A) with *Xho*I (nt 3580); filling in the overhang using Klenow fragment and a mixture of deoxynucleotide triphosphates; digesting with *Hpa*I, a unique site introduced at nt 3202; and recircularizing the DNA using T4 DNA ligase in the presence of NaCl and hexamine cobalt(III) chloride (Rusche and Howard-Flanders, 1985). Clones were initially screened by restriction digestions when possible and then sequenced in the region of the mutation for confirmation. Transcripts from two or more clones of each type were assayed in protoplast infections to test for the presence of additional mutations that might affect phenotypes.

RNA synthesis

Uncapped transcripts were synthesized using T7 RNA polymerase (Pokrovskaya and Gurevich, 1994) from cDNAs that were linearized with *Sma*I, which produces an uncapped version of MCMV gRNA with the exact 3′ terminus. Transcripts were purified and concentrated by three precipitations from 1 M LiCl at 4°C. Template DNA was removed by digestion with RNase-free DNase I (Worthington Biochemicals, Freehold, NJ) either immediately after the transcription reaction or after the first LiCl precipitation. RNA was dissolved in sterile deionized water, quantified, and stored at −20°C.

Protoplast inoculations

Protoplasts were isolated from a BMS maize suspension culture and inoculated with 10 or 15 μg of transcript RNAs (Scheets *et al.,* 1993). Equal aliquots of $1–1.5 \times 10^6$ protoplasts were used in different experiments. Samples for CP and RNA analyses were taken at 0, 24, and 48 h p.i. for most experiments. CP samples were immediately stored at −20°C, whereas RNA samples were lysed with two volumes of 6 M guanidinium thiocyanate, 37.5 mM sodium citrate, pH 7, 0.75% sodium sarkosyl, and 0.15 M 2-mercaptoethanol before storage at −20°C. RNA was isolated (Chomczynski and Sacchi, 1987), dissolved in either sterile deionized water or 90% formamide, and stored at −20°C.

CP detection

CP was detected using rabbit anti-MCMV antiserum in a protein A sandwich-ELISA (PAS-ELISA) as before (Scheets *et al.,* 1993). Samples were assayed in duplicate using *p*-nitrophenyl phosphate as substrate, which was monitored for absorbance at 405 nm.

Northern blots

Electrophoresis and hybridizations were performed similar to previous conditions (Scheets, 1998). RNA from mock-inoculated or infected plants or protoplasts was denatured in a formaldehyde/formamide solution by heating, separated on a 0.9% or 1% agarose gel, and transferred to Magnagraph (MSI) membrane. After UV cross-linking, the membranes were prehybridized at 60°C and hybridized using a ^{32}P-labeled riboprobe synthesized with T3 RNA polymerase. *Nco*I-linearized BS-*Spe*I, a cDNA of the 3′ terminal 253 nt of MCMV (Scheets *et al.,* 1993), produced a 159-nt probe, and *Bam*HI-linearized pMCM602 (Nutter *et al.,* 1989) produced a 1.1-kb probe of the 5′ end of MCMV. Washed blots were wrapped in plastic and exposed to a PhosphorImager (Bio-Rad, Hercules, CA).

RNA isolation from plants

Maize (Pioneer Hi-Bred 3168) was inoculated with MCMV-Kansas. Total RNA was isolated from healthy and

MCMV-infected plants as previously described (Scheets, 1998). DsRNA was isolated from healthy and MCMV-infected plants using Method 2 of Morris and Dodds (1979). DsRNA was separated on a nondenaturing 1% agarose gel in buffer containing 0.5 μg of ethidium bromide and photographed.

Primer extension

The 5′ ends of sgRNAs were mapped by primer extension with Moloney murine leukemia virus reverse transcriptase using a modification of the method of Wang and Simon (1997). Total RNA (0.5 μg) from healthy or MCMV-infected maize plants or 0.1 μg of pMCM41 transcript was annealed to 1 pmol of a ^{33}P-labeled oligonucleotide (p7sg RO, p25 RO, or 3′sg RO from Table 1) and reacted as described using 100 U of Superscript II (Life Technologies, Grand Island, NY) for extension. One fifteenth of each primer extension reaction was separated on an 8% polyacrylamide–8 M urea sequencing gel with dideoxy-termination sequencing reactions (Sequenase, Amersham, Arlington Heights, IL) of pMCM41 using the same radiolabeled primers. The dried gels were exposed to a PhosphorImager.

5′ RACE

Total RNA and dsRNA from MCMV-infected maize were used for 5′ RACE. DsRNA was separated on a nondenaturing 1% agarose gel, and the smallest dsRNA was isolated using a QIAquick Gel Extraction kit (Qiagen, Studio City, CA). The 5′ end was amplified using a 5′ RACE System (GIBCO BRL, Grand Island, NY) according to the manufacturer's directions except denaturation preceding reverse transcription was performed by heating for 3 min at 95°C for dsRNA. After reverse transcription using oligonucleotide OSU4 (Table 1), the cDNA was amplified using 5′ RACE Anchor Primer (CUACUACUACUAGGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG) and GSP1 (Table 1). After digestion with *Sal*I (underlined sequence above), the DNA was cloned into Bluescript SK$^+$ cut with *Xho*I and *Sma*I.

RNA secondary structure predictions

The M-FOLD program (Zuker 1989) in the Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, WI, and the STAR program, Version 4.4 (Gultyaev *et al.,* 1995), were used for RNA secondary structure predictions.

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